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1 **The effects of small litter rearing on ovarian function at puberty and adulthood**
2 **in the rat**

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Abstract

Rearing rats in small litters leads to obesity and reproductive dysfunction. We investigated the effects of rearing female rats in small litters on various reproductive parameters during puberty and into adulthood, and examined the possible involvement of local ovarian sympathetic nerve activity. The litter size was adjusted on postnatal day one to four pups per dam for the small litters and 12 pups per dam for the normal litters. Vaginal opening was recorded, and estrous cyclicity was monitored daily immediately post puberty for 14 days and again at 8-9 weeks of age. At the time of puberty and 10 weeks of age, the ovaries were collected. The number of different types of follicles was counted and the thickness of the theca interna of the largest antral follicles was measured. Ovarian sympathetic nerve activity was assessed immunohistochemically by measuring levels of ovarian nerve growth factor receptor (p75NGFR) and tyrosine hydroxylase (TH). In rats reared in small litters, there was a significant advancement of puberty and disruption of estrous cyclicity immediately post puberty. The number of antral follicles increased in the small litter reared rats at the time of puberty compared with their controls. The thickness of the theca interna increased and the expression profiles of ovarian p75NGFR and TH increased in small litter reared rats at puberty, but this did not persist into adulthood. These data suggest that rearing rats in small litters leads to irregular reproductive cycles, which might involve increased local ovarian sympathetic nerve activity.

Key Words: ovary, obesity, puberty, follicle, sympathetic.

1. Introduction

Postnatal overfeeding induced by rearing animals in small litters results in a dramatic increase in body weight gain and programs for overweightness and persistent hyperphagia in adulthood, even though a standard diet was provided after weaning [1]. It is well established that reproductive function is gated by the state of energy reserves of the organism. The timing of puberty in mammals is tightly coupled to the animals' nutritional and metabolic state. Food restriction in female rats delays puberty [2,3], while female rats reared in small litters have been shown to display early puberty onset [1,4]. Obesity also affects female reproductive function by affecting spontaneous ovulation in humans and animals. The relationship between excess body fat and reproductive disorders in women appears to be stronger for early-onset obesity during their life, particularly during adolescence [5]. There is evidence that in adolescent and young women, the age of onset of obesity and of menstrual irregularities and oligo-anovulation are significantly correlated [5,6]. The prevalence of obesity in women with PCOS appears to be much greater than expected in the general population. Studies in the cafeteria diet-overfed [7] and genetically obese Zucker female rats [8] have shown that these animals display a disruption of estrous cyclicity as well as obesity in adulthood. However, the mechanism of how obesity or overweight link to ovarian dysfunction is not established and there is a sparse literature on the effect of rearing in small litters on ovarian activity in the rat. Rats reared in small litters has been shown to accumulate more noradrenaline in the heart than animals reared in larger litters [9], with cardiac noradrenaline

concentrations inversely related to litter size at 40 days of age [10]. It was shown that noradrenaline levels were also increased in hypothalamus [11] and ovary in the rat [12] during puberty. Abnormally increased noradrenaline levels [13] and tyrosine hydroxylase (TH) immuno-staining in ovary has been demonstrated to cause ovarian dysfunction such as early vaginal opening, disrupted estrous cyclicity and appearance of cystic follicle in the polycystic ovary (PCO) rat [14]. Transection of the superior ovarian nerve decreases noradrenergic innervation of the ovary and restores estrous cyclicity and ovulation in this model [15]. Moreover, increased density of catecholamine nerves has been observed in the ovaries of PCO patients [16] and the marked effectiveness of ovarian wedge resection to initiate ovulatory cycles in PCO patients further supports the importance of sympathetic activity on ovarian function [17,18]. However, there is no literature on whether the ovarian sympathetic nerve activity was affected in rats reared in small litters.

The aim of this study is to test the hypothesis that rearing female rats in small litters as a model of postnatal overnutrition, which advances puberty, is associated with ovarian dysfunction at puberty and in adulthood, as manifested by alterations in ovarian cyclicity and morphology and expression of ovarian nerve growth factor receptor (p75NGFR) and TH; markers of sympathetic nerve activity.

2. Materials and Methods

2.1 Animal procedure

Pregnant Sprague-Dawley rats (Charles River, Manston, UK) were housed under controlled conditions (12 h of light and 12 h of darkness with lights on at 07:00 h and

a controlled ambient temperature of $22 \pm 2^{\circ}\text{C}$) and supplied with *ad libitum* food and water. On postnatal day (pnd) 1 (birth, pnd 0) litter size was adjusted to 4 pups per dam for the small litter reared group ($n = 7$), and to 12 pups per dam for the normal litter reared group ($n = 6$). At least 1 male pup was included in each litter. The dams were singly housed during pregnancy and lactation, with weaning on pnd 21. Post weaning, 4-6 pups were housed in the same cage until they reached 10 weeks of age. They had free access to water and food. All animal procedures were conducted under the British Home Office Animal Scientific Procedure Act 1986 (Project Licence 0671) and in accordance with accepted standards of the local ethical review committee.

2.2 Puberty onset and estrous cyclicity monitoring

Animals were monitored daily for vaginal opening from pnd 28. Once vaginal opening occurred, vaginal smears were taken and monitored daily for 2 consecutive weeks and again at 8-9 weeks of age. The criteria for normal estrous cyclicity were the same as we described previously [19]. Animals were weighed weekly until the end of the experiment.

2.3 Ovarian morphology

Pubertal ovaries were collected on pnd 42 and adult ovaries were collected at diestrus at the age of 10 weeks. The right ovary was cleaned of fat tissue, weighed and fixed in 10% formaldehyde buffer for 20 h at room temperature. Wax-embedded ovaries were sectioned longitudinally at $4\mu\text{m}$ and mounted on glass slides. For counting the number of the different types of follicles, every other section was stained with haematoxylin-eosin and subjected to analysis. Only follicles in which the nucleus of

the oocyte was visible were counted [20]. The thickness of the theca interna layer of the largest follicle was determined in every tenth section (six sections per ovary) with a calibrated scale bar in the microscope. The sections were examined under a light microscope (Zeiss Axioskop 2 plus, Oberkochen, Germany) with an image analysis system (Axiovision 2.05; Zeiss) by two independent investigators blind to the treatment group. The follicles were classified as following: primordial -follicles with oocytes surrounded by one layer of flattened pregranulosa cells; primary-follicles with oocytes surrounded by no more than two layers of cuboidal granulosa cells; preantral follicles without any antral cavity and with two or more layers of granulosa cells; antral-follicles with apparent cavity [20].

2.4 Immunohistochemistry

Immunohistochemical staining for p75NGFR and TH were performed on 4µm paraffin embedded sections from ovaries of 6- and 10-week-old rats. The sections were deparaffinised in xylene, hydrated in descending concentration of ethanol and incubated with 0.3% Triton X-100 (Sigma-Aldrich) in PBS to increase permeability before incubated with 0.3% H₂O₂ to inhibit endogenous peroxidases and with non-immune goat serum (Vector Laboratories, Burlingame, CA, USA) in PBS to reduce background staining. The sections were then incubated in 1:1000 monoclonal mouse anti-p75NGFR primary antibody (Chemicon International, Temecula, CA, USA) containing 2% normal goat serum at 4° C for 24 h. (or 1:1500 polyclonal rabbit anti-TH primary antibody containing 2% normal goat serum at 4°C for 36 h). The sections were then rinsed in 1:150 biotinylated goat anti-mouse IgG (Vector

Laboratories) for 90 min (or 1:300 goat anti-mouse IgG for 120 min) at room temperature followed by 1:200 conjugated avidin-biotin complex (Vector Laboratories) for additional 45 min at room temperature. Visualisation of p75NGFR and TH immunoreactivity was achieved using the 3',3'-diaminobenzidine (Sigma-Aldrich). The sections were counterstained with 10% haematoxylin and coverslipped with DPX. Negative controls were run by incubating adjacent sections with non-immune serum and omission the primary antibody. The sections from the normal litters and small litters were run together in the same batch. Immunohistochemical detection of rat spinal cord and adrenal were included as a positive control for p75NGFR and TH in each batch of experiments. In each immunohistochemical experiment, two control sections from the same ovary tissue were included as immunostaining quality controls for the variation between different batches of experiments.

Six sections per ovary from each rat were used for immunostainings and five fields of vision from each section were analyzed under optical microscope. Semi-quantitative analyses of immunostaining intensity for p75NGFR and TH were carried out on a Zeiss AxioVision microscope image system (Zeiss). All analyses were performed on coded slides by two independent investigators blinded to the treatment groups. The intensity of immunostaining of p75NGFR and TH were assessed by using a modification of a semi-quantitative *H*-Score method described by Akercan *et al* [21]. Semi-quantitative immunohistochemical *H*-score values were calculated from the intensity and percentage of cells staining at each intensity. Intensities were classified as 0 (no staining), +1 (weak staining), +2 (weak-moderate staining), +3 (moderate

staining), +4 (strong staining), +5 (very strong staining). For each slide, the *H*-Score was calculated using the equation: $\sum(I \times PC)$, where *I* and *PC* represent intensity and percentage of cells that stain at each intensity respectively. Data were expressed as mean \pm S.E.M. The different groups were analyzed by the Mann-Whitney *U* test. *P* values <0.05 were considered statistically significant.

2.5 Statistical analysis

Comparisons between the small litter and normal litter reared groups in terms of body weight, vaginal opening and first vaginal estrus were made by subjecting data to one-way ANOVA followed by Dunnett's test. The percentage of normal estrous cycles between groups was compared using X^2 test. Comparisons between groups on thickness of the theca interna layer were made by subjecting data to the Mann-Whitney *U* test. All data are shown as mean \pm S.E.M. *P* values <0.05 were considered statistically significant.

3. Results

3.1 Rearing animals in small litters advanced vaginal opening and first vaginal estrus

The gain in body weight was significantly greater in small litter compared with normal litter reared rats throughout development, although there was no difference at week one postpartum. The significant difference in body weight persisted into adulthood (Fig.1).

For the timing of puberty, rearing female rats in small litters significantly advanced

both the day of vaginal opening (small litter: 34.3 ± 0.3 , n=20; normal litter: 36.3 ± 0.3 , n=22; $P < 0.05$) and the day of first vaginal estrus (small litter: 34.8 ± 0.5 , n=20; normal litter: 36.5 ± 0.3 , n=22; $P < 0.05$).

3.2 Rearing animals in small litters disrupted estrous cyclicity at puberty without persisting into adulthood

Representative examples of estrous cycles in each group are illustrated in Fig. 2A and B. The majority (66.4%) of rats reared in the normal litter group showed a typical 4-5 day estrous cycles at puberty, and this percentage increased to 77.8% in adulthood (Fig. 2C). In rats reared in small litters, only 35.0% displayed normal cyclicity at puberty, which improved to 65.0% in adulthood. The percentage of normal estrous cycles was significantly lower in the small litter reared group at puberty (Fig. 2C).

3.3 Effect of rearing animals in small litters on ovarian weight and morphology at puberty and in adulthood

The mean ovarian weight at puberty (pnd 42) for the normal litter and small litter reared groups was 36.80 ± 3.81 mg (n=20) and 35.53 ± 3.02 mg (mean \pm S.E.M, n=22) respectively. At adulthood (10 weeks), ovarian weight for the normal litter and small litter reared animals was 71.41 ± 8.32 mg (n=20) and 72.23 ± 10.24 mg (mean \pm S.E.M, n=22), respectively. There was no significant difference in ovarian weight between the small and normal litter reared rats either at puberty or in adulthood. There was no significant difference in the mean number of primordial, primary or preantral follicles, or corpora lutea either at puberty or adulthood (Table 1). However, the number of antral follicles was increased at puberty in the small litter reared rats compared with

the normal litter reared animals (Table 1).

The thickness of the theca interna layer of the largest follicle was significantly increased in ovaries from the small litter reared group at puberty (Fig. 3A, B, C), but this difference did not persist into adulthood (Fig. 3D).

3.4 Effect of rearing animals in small litters on sympathetic tone in the ovary of pubertal and adult rats.

The immunohistochemical staining for p75NGFR was generally confined to the theca cells, especially theca interna cells of growing follicles (preantral and antral follicles; Fig. 4A-D). Neither primordial nor primary follicles had specific staining for p75NGFR. There was no difference in the staining pattern between ovaries from the small litter reared and normal litter reared groups. However, the intensity of staining for p75NGFR in preantral and antral follicles was enhanced at puberty in the small litter compared with the normal litter reared control animals (Fig. 4E). In adulthood (diestrus) this difference was no longer evident.

The immunostaining for TH was distributed in theca cells of preantral and antral follicles, corpora lutea, and interstitial tissue. The staining pattern for TH in ovaries from rats reared in small litters was the same as that for normal litter controls. However, the staining intensity for TH (theca cells) in preantral follicles was enhanced in ovaries from the small litter reared group at puberty (Fig. 5A-E), compared with the normal litter controls (Fig. 5E), whereas in adulthood (diestrus) this difference was no longer evident.

4. Discussion

This study demonstrates that rearing female rats in small litters can have effects on ovarian morphology and reproductive function at puberty, without persisting into adulthood. Not only were markers of puberty onset (vaginal opening and first vaginal estrus) advanced, but in addition, estrous cyclicity was disrupted at puberty. The disruption to estrous cyclicity was characterized by a persistent or prolongation of vaginal estrus. A remarkable increase in the number of antral follicles was observed in the ovaries of the small litter reared rats compared with their controls at puberty. However, at the adult stage this difference was no longer evident. The thickness of the theca interna layer and the markers of sympathetic tone, including p75NGFR and TH immunoreactivity, in the ovary were increased at the time of puberty in rats from the small litter reared group, but were not evident in adulthood. In our study, postnatal overfeeding by rearing rats in small litters resulted in obesity throughout life, which is in accordance with other studies [22]. Puberty onset is closely correlated with preweaning nutrition and body weight gain [23]. As expected, there is a strong correlation between body weight and age at vaginal opening as previously observed [1,4]. Overnutrition due to small litter rearing as in the present study, or high fat diet feeding, induces a persistent increase in body weight gain and advances puberty [1,4, 24]. Conversely, negative energy balance due to excess exercise or food restriction delays puberty [4,25]. Although the underlying mechanism controlling the timing of puberty onset which is influenced by metabolic cues and nutritional status remains undefined, it appears that appropriate body weight and adiposity are necessary for

puberty to occur [23,26]. Leptin and kisspeptin are affected by adiposity levels and unequivocally required for the onset of puberty. Postnatal overnutrition by rearing rats in small litters is associated with higher levels of leptin and increased levels of hypothalamic kisspeptin mRNA expression at puberty [1], although others report no affect of small litter rearing on hypothalamic levels of kisspeptin expression at puberty [4]. Complex changes in the regulatory mechanisms of the hypothalamic-pituitary-gonadal axis with overnutrition, may attribute to the earlier accelerated GnRH pulse generator frequency to advance puberty [24].

The finding of abnormal estrous cyclicity at puberty in the small litter reared rats suggests abnormal follicular development and ovarian steroid production. Although the impact of postnatal overnutrition, by rearing in small litters, on ovarian follicular morphology has not previously been studied in the rat, an increase in the number of the small antral follicle in ovaries of young adult rhesus monkeys fed a Western-style diet was reported recently [27]. We found in the current study that the number of antral follicles was markedly increased, by more than 100%, at puberty in the small litter reared rats. However, this difference was lost in the adult rat and may therefore explain why a significantly higher number of irregular estrus cycles were detected only immediately after puberty, but not at the young adult stage in the small litter reared animals. This is consistent with a recent study showing that rearing in small litters did not alter the duration of the estrous cycles in the young adult female rats [28]. The mechanism for the increased number of antral follicles in the ovary of the small litter reared rat could be a direct malfunction of the reproductive system

induced by overnutrition at the level of the hypothalamus, pituitary, or ovary either independently or in combination. It is generally accepted that the rise in FSH controls the dynamics of small antral follicle growth and the mid-cycle LH surge stimulates ovulation. It has been reported that postnatal overnutrition by rearing rats in small litters increased FSH levels, whilst reducing circulating estradiol levels [28]. Similarly, pituitary expression of FSH β mRNA was elevated in small litter reared rats [28]. However, one of the most striking features of women with PCOS is the presence of numerous antral follicles accompanied with an increased LH pulse frequency [29]. Previous studies have shown an increase in basal LH levels in small litter reared rats [1], which may impact on other hormonal signals that may in turn attribute to changes in follicle development, luteal formation or sex steroid production in the ovary. Very recently, increased numbers of small antral follicles owing to presence of more atretic follicles, and alterations in the transcriptome of the small antral follicle were detected in young adult monkeys fed with high fat/fructose diet [27], which are similar to those observed in women with obesity and/or PCOS. Furthermore, decreased ovarian reserve, dysregulation of mitochondrial biogenesis, and increased lipid peroxidation has been reported in mouse offspring exposed to an obesogenic maternal diet (high fat/high sugar) [30].

The effects on ovarian morphology and sympathetic activity observed immediately post-puberty in rats reared in small litters is presumably regulated by local intra-ovarian mechanisms and/or changes in the activity of neural inputs to the ovary. A single injection of estradiol valerate to neonatal rats was demonstrated to disrupt

estrous cyclicity and induce the development of PCO that was causally related to an increase in ovarian noradrenaline concentration [13]. It is interesting to note that this treatment with estradiol valerate did not increase noradrenaline concentration in the celiac ganglion, the central sympathetic innervation to the ovary [13]. It has been shown that the pre-pubertal increase in follicular noradrenaline regulates the follicular response to gonadotrophins and ovulation [31,32], while denervation of the ovary results in a delay of follicular development and puberty onset [20]. Increased sympathetic innervation of the ovary has been shown to disrupt estrous cyclicity and impair follicular maturation [33,34].

The increased ovarian sympathetic tone, indicated by the elevated immunoreactivity for TH and p75NGFR especially in the theca interna, in the small litter reared rats may underlying the disruption of estrous cyclicity in the immediate post-puberty period observed in the present study. An up-regulation of sympathetic tone indicated by augmented production of ovarian nerve growth factor was shown to contribute to the formation of ovarian cysts in a rat PCO model induced by estradiol valerate [34,35]. Furthermore, estradiol valerate not only increases intraovarian synthesis of nerve growth factor but its receptor p75NGFR, which was abundantly expressed in both the thecal compartment and nerve fibers of ovaries [35].

Although ovarian sympathetic innervation is important for follicular development, steroid secretion and ovarian function [20,36], abnormal increased sympathetic activity in the ovary has also been shown to precede disrupted estrous cyclicity, reduce ovulation and the appearance of cystic ovaries in stress animal models [37]. In

the present study, the number of antral follicles and the thickness of the theca interna layer of the largest follicle were increased in the immediate post-puberty period in the small litter reared animals. This may be due to the increased ovarian sympathetic tone since previous studies using chronic intermittent cold stress, which classically enhances ovarian sympathetic activity, showed increased thickness of the theca layer in the rat ovary [37].

Human polycystic ovaries have been found to have an increased sympathetic innervation [38] and ovarian wedge resection, especially when compromising the hilum, the point of nerve entry into the ovary, has been shown to be effective in PCOS patients unresponsive to standard treatment with clomiphene citrate [17]. Furthermore, sympathetic hyperactivity in PCOS patients has been detected by indirect [39] and direct measurements of sympathetic nerve mediated ovarian steroid secretion and intraovarian neurotrophic influences on follicular development could have a function in the development of PCOS [16]. Recently, it was shown that reducing sympathetic outflow by exercise or acupuncture decreased the thickened theca interna layer in the dihydrotestosterone-induced PCO rat model [40]. Therefore, the increased markers of ovarian sympathetic activity observed in the present study may underlie the altered ovarian morphology and disruption of estrous cyclicity in the small litter reared rats at puberty. Once these animals reached adulthood, the abnormal estrous cycles and thickened theca interna layer were no longer evident, which correlates with the disappearance of the increased ovarian sympathetic tone markers at this stage. Similarly, in girls PCOS commonly starts at the time of puberty, but symptoms

usually relieve or improved as they reach adulthood. The results from our study may help to understand the mechanism underlying PCOS in adolescence.

In conclusion, this study shows that rearing rats in small litters, as a model of overnutrition, leads to ovarian dysfunction at puberty, especially disruption of estrous cyclicity, increased numbers of antral follicles and thickening on the theca interna, which might be associated with increased local ovarian sympathetic tone similar to what is observed in women with polycystic ovary.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends

Figure 1. Effects of rearing animals in small litters on body weight. The animals reared in small litters show a significant increase in body weight, which persists into adulthood. * $P < 0.05$ versus animals reared in normal litter ($n = 20-22$ pups per group).

Figure 2. Rearing rats in small litters disrupted estrous cyclicity immediately post-puberty, with improvement in adulthood. Representative examples of estrous cyclicity are illustrated in (A) and (B). The percentage of normal estrous cycles was decreased at puberty but improved in adulthood in rats reared in small litters compared with normal litter size controls (C). P, proestrus; E, estrus; M, metestrus; D, diestrus. * $P < 0.05$ versus animals reared in normal litters as control ($n = 20-22$ pups per group).

Figure 3 Effects of rearing rats in small litters on the thickness of the theca interna layer of the largest follicle in pubertal and adult ovaries. Representative examples of the theca interna layer of the largest follicle in pubertal ovaries are illustrated in A (small litter) and B (normal litter). The thickness of the theca interna layer of the largest follicles was increased at diestrus in rats reared in small litters at puberty (C), but at adulthood (D). CL, corpus luteum; GC, granulosa cell; TC, theca cell; arrows show the theca interna layer. Photomicrographs illustrate diestrus stage of estrous cycle. Scale bar = 50 μm . * $P < 0.05$ versus animals reared in normal litters as control ($n = 10-11$ pups per group).

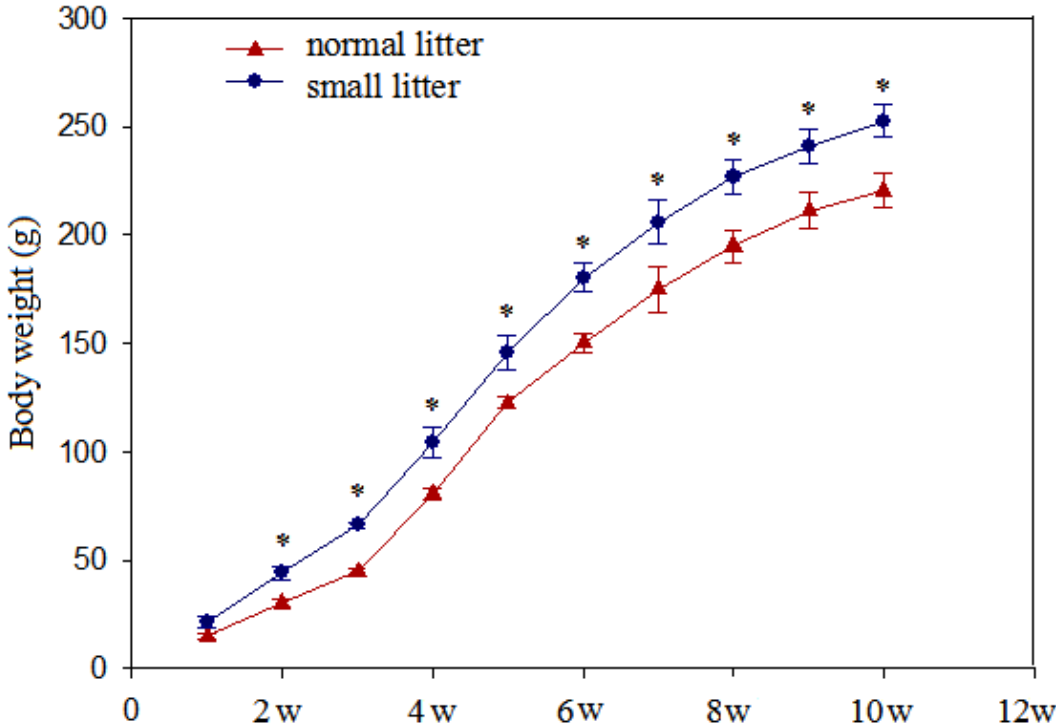
Figure 4 Effects of rearing animals in small litter on the immunoreactivity of p75NGFR in theca interna cells of the pubertal ovary. Representative examples illustrating the expression of p75NGFR protein in theca interna cells of pubertal ovaries from normal litter and small litter reared rats. Theca cells, especially the theca interna (arrows) of growing follicles contain low but detectable levels of p75NGFR protein in normal litter controls (A). The immunostaining for p75NGFR was significantly enhanced in the rats reared from the small litters (B). C, shows a higher magnification of the boxed area in B. D, shows the control section incubated in absence of primary antibody. H-Scores of immunostaining intensity for p75NGFR in theca interna cells are summarised in E. GC, granulosa cell; TCI, theca interna cell; TCE, theca externa cell. Photomicrographs illustrate diestrus stage of estrous cycle. Scale bars: A, B, D=50 μ m; C=25 μ m. * P <0.05 versus animals reared in normal litters as control (n =10-11 pups per group).

Figure 5 Effects of rearing animals in small litter on the immunoreactivity of TH in theca interna cells of the pubertal ovary. Representative examples illustrating the expression of TH protein in theca cells of pubertal ovaries from both small litter and normal litter reared control rats. Theca cells (arrows) of the growing follicles contain low but detectable levels of TH protein in normal litter rats (A). The immunostaining for TH was significantly enhanced in the small litter reared rats (B). C, shows a higher magnification of the boxed area in A. D, shows the control section incubated in absence of primary antibody. H-Scores of immuno-staining intensity for p75NGFR in

502 theca interna cells are summarised in E. The staining intensity was significantly
503 enhanced in the preantral follicles from small litter reared rats compared with normal
504 litter size controls. GC, granulosa cell; TC, theca cell; CL, corpus luteum. Scale bars:
505 A, B, D=50 μm ; C=25 μm . * $P<0.05$ versus normal litter reared animals as control
506 ($n=10-11$ pups per group).

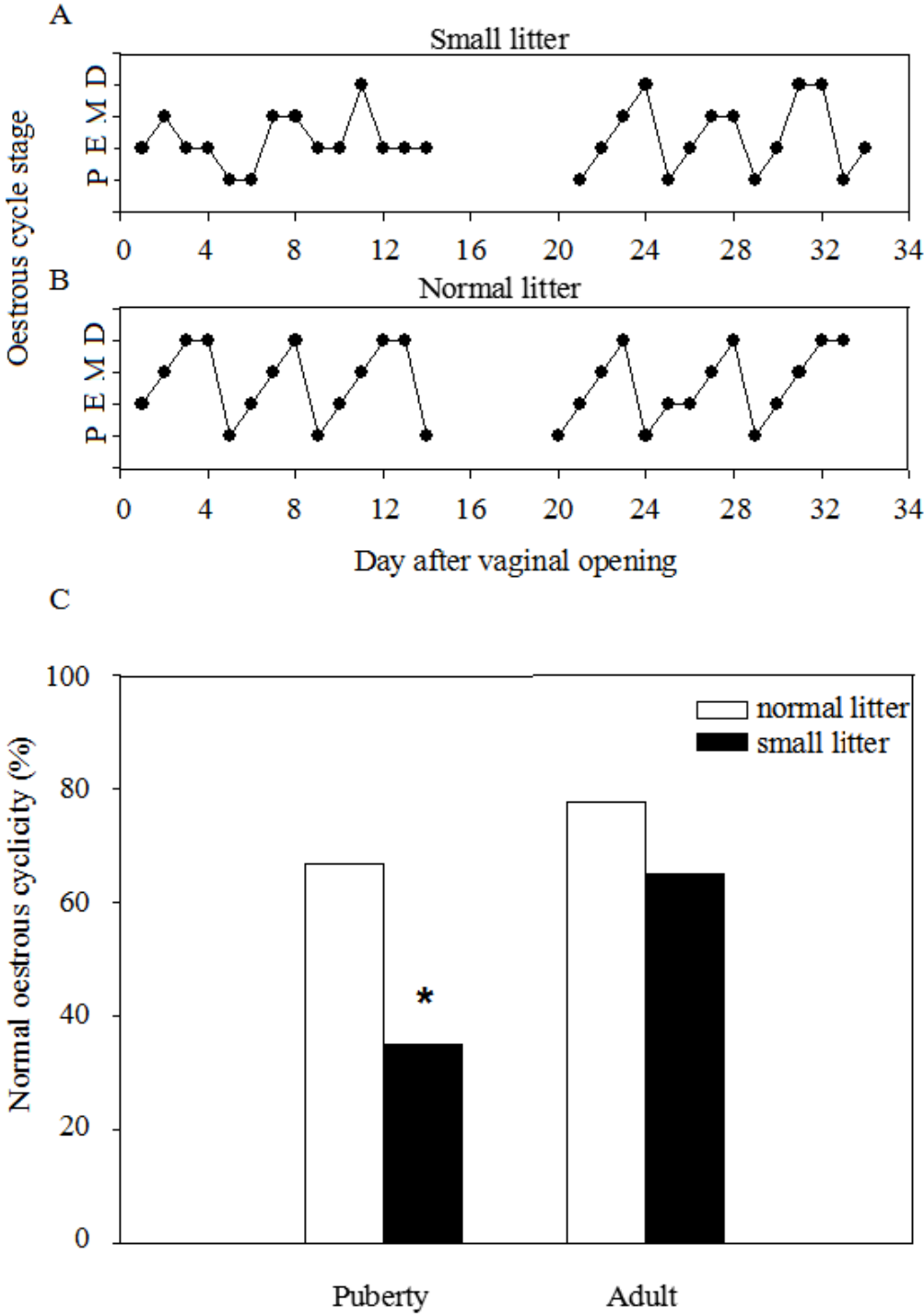
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508 **Figure 1**

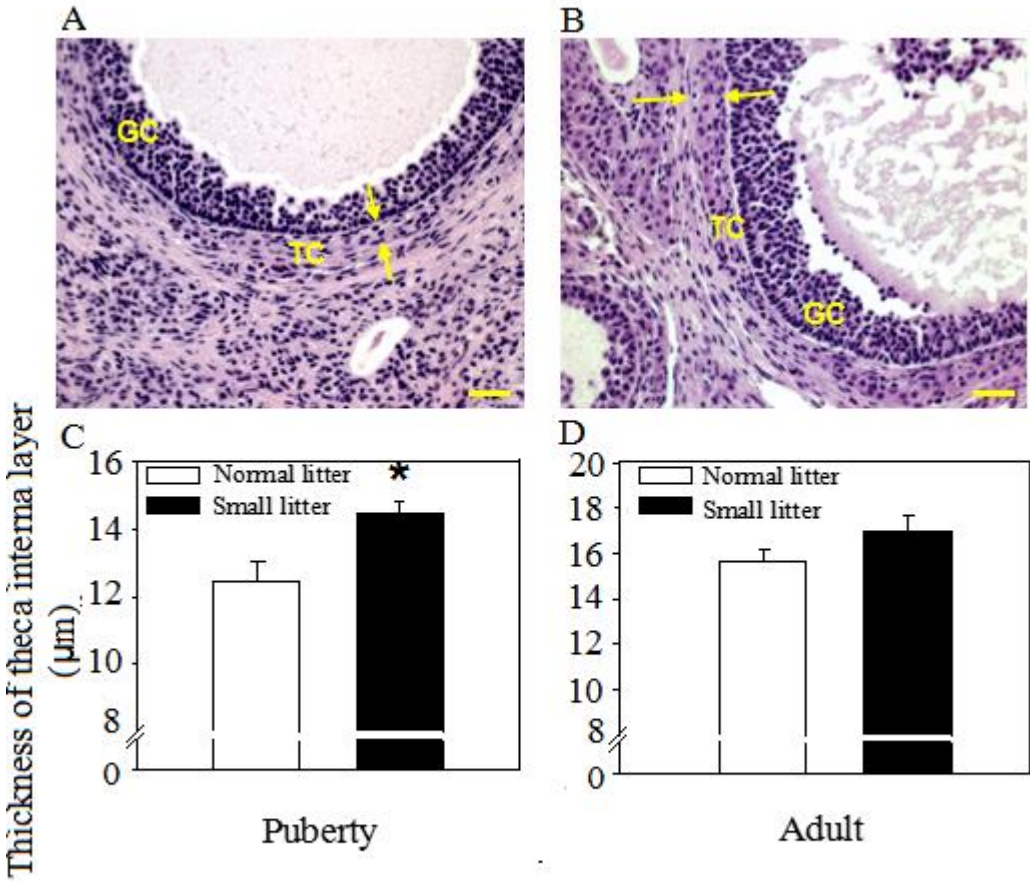


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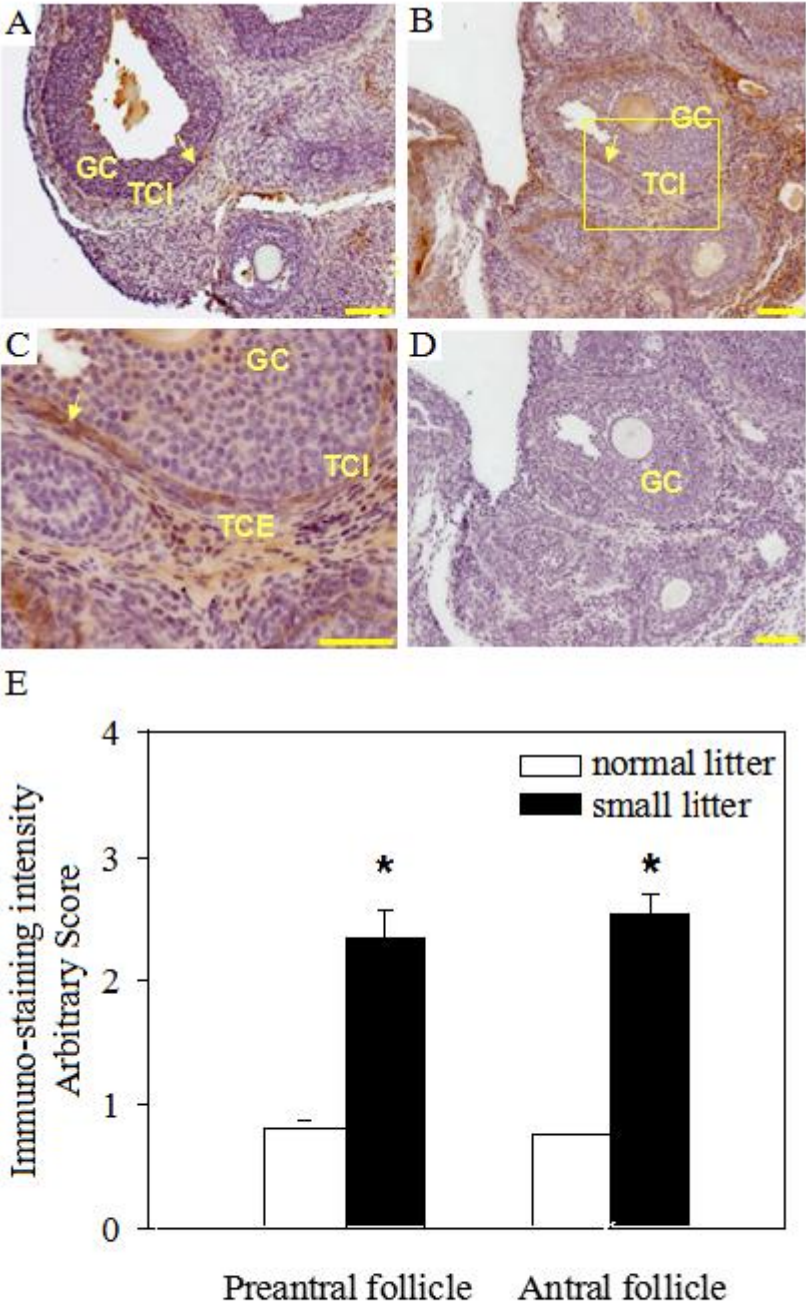
514 **Figure 3**



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517 **Figure 4**



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